UNIVERSIDADE ESTADUAL DE CAMPINAS - UNICAMP

FACULDADE DE ODONTOLOGIA DE PIRACICABA - FOP

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"Efeito antimicrobiano *in vitro* da *Mikania laevigata* e *Mikania glomerata* sobre estreptococos do grupo mutans".

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, no Programa de Pós-Graduação em Odontologia, para obtenção do título de Mestre em Odontologia, Área de Farmacologia, Anestesiologia e Terapêutica.

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SUMÁRIO

RESUMO

O objetivo geral do trabalho foi avaliar o efeito antimicrobiano da *Mikania laevigata* e *Mikania glomerata* (guaco) sobre os estreptococos do grupo mutans. Portanto, foram realizados 2 estudos, sendo o objetivo do estudo 1 analisar a composição química e o efeito antimicrobiano dos extratos e frações da *Mikania laevigata* e da *Mikania glomerata* sobre o crescimento bacteriano e a aderência celular dos estreptococos do grupo mutans. O objetivo do estudo 2 foi avaliar o efeito dos compostos isolados da *Mikania* sobre a viabilidade das células dos estreptococos do grupo mutans em biofilme (BF) e durante sua formação. Para o estudo 1 foram feitos extratos hidroetanólicos, frações hexânicas e de acetato de etila, sendo analisados quanto a sua composição química por cromatografia gasosa acoplada a espectrometria de massa (CG/MS). Os extratos e frações foram avaliados microbiologicamente por meio das determinações da concentração inibitória mínima (CIM), concentração bactericida mínima (CBM) e da inibição da aderência celular à superfície de vidro dos microrganismos do grupo mutans, utilizando os métodos descritos por Koo *et al.,* 1999. As frações hexânicas da *M. laevigata* e *M. glomerata* apresentou os melhores resultados antimicrobianos dentre as outras frações testadas (CIM entre 12,5 a 100 μg/mL e CBM entre 12,5 μg/mL a 400 μg/mL) e inibição da aderência celular entre 3,125 e 12,5 μg/mL). Para o estudo 2, os compostos ativos da fração hexânica da *Mikania* foram isolados e identificados por métodos espectroscópicos, sendo isolados 16 compostos diferentes como o ácido cupressênico, ácido diterpênico, ácido caurenóico, cumarina e ácido glandifórico, dentre outros. Entretanto, somente os ácidos cupressênico, diterpênico e caurenóico apresentaram atividade antimicrobiana nos testes de CIM e CBM, sendo esses 3 ácidos os compostos testados (concentrações 1, 10 e 100 vezes a CIM) sobre a viabilidade (*time-kill)* e inibição da formação do biofilme (IFB) de *Streptococcus mutans* UA 159 e *S. sobrinus* 6715. Os BFs foram formados sobre lâminas de vidro obtendo-se uma concentração final de 10^8 ufc/BF. Os resultados de *time-kill* e IFB foram expressos em log N/N_0, sendo N_0 as ufc do controle negativo e N as ufc formadas após os tratamentos. Para o teste IFB foram realizadas as determinações de

polissacarídeos insolúveis e proteínas totais desse biofilmes. Os testes foram realizados em 6 duplicatas. Os resultados foram submetidos à análise estatística ANOVA e Tukey-Kramer HSD ($p<0,05$). Os ácidos diterpênico e cupressênico apresentaram atividade bactericida no teste de viabilidade bacteriana nas concentrações de 100 vezes a CIM para *S. mutans*, e o ácido caurenóico reduziu o número de células viáveis, entretanto não sendo bactericida. Nenhum dos compostos testados apresentou atividade bactericida para *S. sobrinus* após 4 h de tratamento. No teste IFB, nenhum dos extratos testados apresentou atividade bactericida para *S. mutans* e somente a fração hexânica 1250 μg/mL foi bactericida para *S. sobrinus.* O ácido diterpênico e o ácido cupressênico foram os únicos compostos efetivos reduzindo a produção de polissacarídeos insolúveis e proteínas totais nos biofilmes, sendo que o ácido diterpênico apresentou os melhores efeitos antimicrobianos em modelo de biofilme. Desta forma, concluímos que os ácidos cupressênico e diterpênico da *Mikania* possuem potencial antimicrobiano, atuando sobre o biofilme dental em baixa concentração, demonstrando serem promissores agentes anti-cárie e anti-placa.

ABSTRACT

The overall aim of this study was to evaluate the antimicrobial effect of *Mikania laevigata* and *Mikania glomerata* (guaco) on mutans streptococci. Therefore, two studies were carried out. The aim of study 1 was to analyze the chemical composition and the antimicrobial effect of crude extract and fractions of *M. laevigata* and *M. glomerata* on bacterial growth and cell adherence of mutans streptococci. The aim of study 2 was to evaluate the effect of the isolated compounds of *Mikania* on the population of viable cells of mutans streptococci in biofilm or during its accumulation. In study 1, the composition of crude hydroalcoholic extracts, hexane and ethyl acetate fractions of both *Mikania* were chemically analyzed by Gas Chromatography with was spectroscopy analysis coupled with a Mass Detector (GC-MS). The antimicrobial activity of crude extracts and fractions were assessed by determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and inhibition of cell adherence (Adh) to glass surface of mutans streptococci, as described by Koo *et al*., 1999. The hexane fractions from both plants was the most effective in inhibiting the growth of all the bacterial strains tested (MIC 12.5 to 100 μg/ mL and MBC 12.5 μg/mL to 400 μg/mL) and adherence cells (3.125 e 12. 5 μg/mL). In study 2, the active compounds from hexane fraction of *Mikania* were isolated and identified by spectroscopy methods (NMR ^{1}H and ^{13}C) finding 16 different compounds as cupressenic, diterpenic, kaurenoic, glandifloric acids, coumarin and octadecene, among others. Although, only the cupressenic, diterpenic and kaurenoic acids showed antimicrobial activity for MIC and MBC tests against mutans streptococci, which were tested (concentration 1, 10 e 100 times MIC) on killing assays (*time-kill)* and inhibition of biofilm formation (IBF) of *Streptococcus mutans* UA159 and *S. sobrinus* 6715 biofilms. The biofilms were formed on standard glass microscope slides with TSB/sucrose. The results of time-kill and IBF were expressed in log N/N_0 where N_0 is the number of colony of the negative control and N is the cfu produced after the indicated times of treatment. The biofilms of IBF test were dried and used to determine insoluble polysaccharide by colorimetrically method and total protein concentration in

biofilms based on Lowry method. All of the assays were carried out in six duplicates and statistical analyses were carried out. The data were analysed using Anova and Tukey-Kramer HSD test ($p<0.05$). In the time-kill test, diterpenic and cupressenic acids at concentrations of 100 times MIC, showed bactericidal effects on *S. mutans* biofilm. However, none of the agents tested were bactericidal for the *S. sobrinus* biofilms, showing that kaurenoic acid was the compound that better reduced the number of viable cells after 4 h of treatment. In the IBF, none of the agents tested appeared to be bactericidal for *S. mutans* biofilms and only hexane fraction 1250 μg/mL was bactericidal to *S. sobrinus*. The cupressenic and diterpenic acids were the compounds that effectively reduced the insoluble polysaccharide and total protein in biofilms, revealing the diterpenic acid showed the major antimicrobial effect among the other compounds tested, reducing the viable cells in biofilm model. In conclusion, the cupressenic and diterpenic acids of *Mikania* have relevant antimicrobial activity against mutans streptococci on biofilms at low concentration and are promising anti-caries and anti-plaque agents.

1. INTRODUÇÃO

A cárie e a doença periodontal são as infecções bacterianas mais comuns nos seres humanos (Loesche, 1986), sendo que a placa dental tem sido extensivamente estudada pela sua relação com estas infecções (Gibbons e van Houte, 1975).

Placa dental é um biofilme bacteriano encontrado naturalmente na superfície dos dentes, apresentando composições bacteriana e bioquímica que podem variar em dependência de fatores intrínsecos e extrínsecos (Marsh, 1992).

O biofilme é benéfico ao hospedeiro na medida em que ajuda a prevenir a colonização de microrganismos exógenos e patógenos. Sua composição é relativamente estável apesar da exposição regular a mudanças no ambiente oral. Entretanto esta estabilidade pode deixar de existir quando ocorrem alterações do meio oral, conduzindo a um desequilíbrio da microbiota bucal residente, favorecendo o estabelecimento de uma população microbiana cariogênica (van Houte, 1994; Loesche, 1986; Marsh, 1992, 1994) Subseqüentemente, bactérias colonizadoras secundárias se aderem aos residentes já aderidos (co-agregação) por interações moleculares específicas. Este processo contribui para a determinação do padrão de sucessão bacteriano, formando um biofilme patogênico, também conhecido como "placa dental, doença" ou biofilme patogênico. (Marcote e Lavoie, 1998).

Um dos fatores de desequilíbrio fundamental para o aparecimento de uma placa dental cariogênica é a dieta rica e freqüente de carboidratos fermentáveis, principalmente a sacarose. Esta dieta promove um aumento da proporção de estreptococos do grupo mutans, uma vez que estes microrganismos apresentam algumas vantagens ecológicas quando da presença deste açúcar no meio bucal, permitindo a sua aderência, colonização e posterior acúmulo na superfície lisa do esmalte dental (Hamada e Slade, 1980; Loesche, 1986). Além disso, a fermentação de carboidratos da dieta pelas bactérias, principalmente sacarose, resulta na produção de ácidos e produtos que inicialmente desmineralizam o esmalte e posteriormente a dentina, sendo que, quando

esse processo não é controlado, ocorre à formação das lesões cariosas (Alam *et al*., 2000; Bowden, 1990).

Os estreptococos do grupo mutans, além de serem acidúricos e acidogênicos, não só fermentam a sacarose como, a partir desta, sintetizam glucanos através das enzimas glucosiltransferases - GTFs (Gibbons e van Houte, 1975; Hamada e Slade, 1980). Atualmente, três GTFs distintas, secretadas pelo *Streptococcus mutans*, estão bem caracterizadas tanto bioquimicamente como ao nível molecular: 1) GTF B - codificado pelo gene *gtfB*, que sintetiza glucanos insolúveis em água tendo ligações glicosídicas principais α (1$\rightarrow$3); 2) GTF C - codificado pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis, este último apresentando ligações glicosídicas principais α (1$\rightarrow$6); e 3) GTF D - codificado pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (Loesche, 1986; Hanada e Kuramitsu, 1989). A GTF produzida por *S. sanguinis* (GTF Ss) pode também estar envolvida com o desenvolvimento da placa dental. Os glucanos, principalmente os insolúveis em água, têm sido considerados como os principais fatores de aderência e acúmulo de estreptococos cariogênicos sobre a superfície dental (Hamada e Slade, 1980; Rölla *et al.,* 1983; Tanzer *et al.,* 1985; Schilling, 1992). Em acréscimo, tem sido demonstrado que estes glucanos aumentam a porosidade (Dibdin e Shellis, 1988; van Houte, 1994) bem como causam mudanças na composição inorgânica da matriz da placa (Cury *et al.,* 1997), tornando-a ainda mais cariogênica. Assim, estreptococos do grupo mutans e glucanos são considerados fatores críticos no desenvolvimento da placa dental cariogênica.

Deste modo, estratégias têm sido estudadas no sentido de prevenir a cárie dental e a doença periodontal, seja inibindo o crescimento dos estreptococos do grupo mutans na cavidade bucal ou inibindo a aderência destas bactérias à superfície dos dentes através da inibição da atividade das GTFs, principalmente aquelas responsáveis pela síntese de glucanos insolúveis (GTF B e C), impedindo assim a formação da placa dental. Um agente que eficientemente possuísse propriedades antimicrobianas e inibisse as GTFs seria extremamente desejável para a prevenção destas doenças bucais (Koo *et al.,* 2002).

Nas últimas décadas têm sido observado mundialmente um crescente interesse global no aproveitamento da biodiversidade, particularmente no que se refere às plantas medicinais, que têm sido utilizadas em várias áreas da saúde como uma expressiva forma alternativa de tratamento e prevenção (Lewis e Elvin-Lewis, 1977). O comércio de medicamentos fitoterápicos vem crescendo a uma taxa anual média de 15%, sendo que cerca de 30% dos medicamentos comercializados atualmente são originados direta ou indiretamente de produtos naturais, principalmente de plantas (Farnsworth, 1985; Elisabetsky, 1987).

Esse grande consumo atual de medicamentos fitoterápicos pela população decorre basicamente do fato de que representam formas de terapia mais baratas e /ou naturais que aquelas normalmente oferecidas e preconizadas pela indústria farmacêutica e a medicina alopática. Dentro desse contexto, o aumento do uso destas plantas seria de grande utilidade principalmente nos países em desenvolvimento como o Brasil, que ainda possui grande biodiversidade e tem uma posição privilegiada por possuir cerca de 25% da flora mundial (Farnswotrh, 1985).

Dentro dessa perspectiva de aumento do mercado de medicamentos, a participação das plantas medicinais é, sem dúvida, muito importante, particularmente no que tange o desenvolvimento de medicamentos fitoterápicos e a identificação de novas moléculas ou protótipos básicos para geração de novos medicamentos sintéticos. É verdade também, que muitos constituintes de plantas e /ou seus derivados semi-sintéticos constituem uma parcela apreciável dos medicamentos de ponta recém introduzidos no mercado.

Assim, a procura pela descoberta de novos produtos naturais com atividade antibacteriana para a prevenção de doenças bucais e talvez com menores efeitos adversos quando comparados aos produtos industrializados seriam muito importantes para obtenção de um meio efetivo de controle da formação de um biofilme patogênico. Porém, para avaliar a efetividade destes produtos naturais, são necessárias análises progressivas começando com estudos laboratoriais *in vitro*, passando por modelos de estudo *in vivo* e culminando com os estudos clínicos longitudinais (Ten Cate e Marsh,

1994).

Diante desse contexto, muitos estudos já estão sendo realizados identificando e isolando os princípios ativos presentes nestes extratos naturais, para que estes novos compostos isolados quimicamente, se efetivos na sua ação antimicrobiana, possam ser sintetizados e utilizados no controle da doença cárie.

Como mencionado anteriormente, o uso de plantas medicinais vem crescendo nos últimos anos e isto é devido principalmente as descobertas de suas propriedades biológicas, e entre estas plantas destacam-se as do gênero *Mikania.* Segundo Cruz e Liberalli (1938), a maioria das espécies pertencentes ao gênero *Mikania* possui emprego na terapêutica popular, merecendo destaque especial àquelas conhecidas pelo nome de "guaco" ou "guaco cheiroso". Oliveira *et al.* (1984) afirmam que o guaco é uma das plantas medicinais mais empregadas no Brasil devido aos seus efeitos farmacológicos: antiespasmódico, analgésico (Ruppelt *et al.,* 1991), antiasmático, antibacteriano (Yatsuda, 2001, 2002, 2003), antifúngico (Davino *et al.,* 1989) antiinflamatório (Ruppelt *et al.,* 1991; Pereira *et al.,* 1994), antiprotozoário, antitussígeno, antialérgico (Fierro *et al.,* 1999), broncodilatador, cicatrizante, expectorante, anti-sifílico, antifebril, antiespasmódico e antiúlcera.

As plantas do gênero *Mikania* foram descritas por Willdenow em 1804, recebendo esta nomenclatura em homenagem ao professor Joseph Gottfried Mikan, de Praga. Para o gênero são citadas cerca de 430 espécies, distribuídas principalmente na América Central e do Sul, sendo 200 espécies no Brasil, distribuídas principalmente nas regiões Sul e Sudeste (King e Robinson, 1987). Apesar de apresentar um grande número de espécies, o gênero *Mikania* foi pouco estudado no Brasil. Diversas espécies do gênero *Mikania* são citadas e empregadas em outros países da América do Sul e Central, todas pertencentes à família Compositae (Ritter *et al.,* 1992).

A espécie *Mikania glomerata* Spreng é a única espécie oficializada na 1ª edição da Farmacopéia Brasileira (da Silva, 1929) e foi identificada por Sprengel em 1826, sendo também conhecida por: *Cacalia trilobata* Vell., *M. amara, M. aspera, M. attenuata, M.scansoria* DC., *M. hederaefolia* DC., *Willoughbya glomerata* (Spreng), *Willoughbya moronoa* (Ktze),

sendo popularmente conhecida pelos nomes de guaco, guaco liso, guaco de cheiro, erva de serpente e cipó caatinga (Lucas, 1942; Oliveira *et al.,* 1984). É um subarbusto trepador de ramos lenhosos e de folhas verde-brilhante que, ao serem esfregadas, exalam um forte aroma de baunilha (Vanila) (Oliveira *et al.,* 1984). O seu habitat é nas margens e interiores de matas, adaptando-se muito bem ao cultivo doméstico. Na época da floração torna-se uma planta muito procurada pelas abelhas melíferas. (Ritter *et al.,* 1992; Lucas, 1942; Corrêa, 1952).

A família Compositae, à qual pertence esta espécie, é caracterizada quimicamente pela riqueza em produtos do metabolismo secundário (Gibbs, 1974). A *M. glomerata* apresenta vasta variedade de compostos, tais como ácidos orgânicos, açúcares, materiais corantes, resinas, taninos, cumarina, saponinas, o estigmasterol e um éster alifático, cinamoilgrandiflórico, ácido caurenóico (Oliveira *et al.,* 1984; Santos *et al.,* 1992). No período da floração, agosto a dezembro, ocorre um aumento da concentração dos princípios ativos na planta, decrescendo a produção desses compostos após a floração (Ritter *et al.,* 1992). Dentre os compostos isolados e identificados alguns apresentam atividades farmacológicas destacam-se os diterpenos da classe dos cauranos e cumarinas (Egan *et al.,* 1990; Rosskopf *et al.,* 1992; Pereira *et al.,* 1994; Ruppelt *et al.,* 1991; Davino *et al.,* 1989).

Apesar das vastas aplicações populares, a literatura traz poucos estudos científicos sobre a ação antimicrobiana do "guaco", principalmente em relação a bactérias orais. Em estudos de Alves *et al.* (1995), o extrato bruto das folhas de guaco (*Mikania cordifolia*) demonstrou atividade antiprotozoária (*Trichomonas vaginalis* e *Trypanosoma cruz*), *in vitro,* e ação antibacteriana e anticândida. Trabalhos desenvolvidos por Yatsuda *et al*. (2001 e 2002), demonstraram que o extrato bruto da *M. laevigata* apresenta atividade antimicrobiana e é capaz de inibir *in vitro* a aderência celular de *S. sobrinus.*

Deste modo, devido à carência de informações do potencial antimicrobiano das *M. laevigata* e da *M. glomerata* e de seus compostos químicos sobre os patógenos bucais, o objetivo do presente estudo foi analisar o efeito antimicrobiano *in* vitro da *Mikania*

laevigata e *Mikania glomerata* e seus compostos sobre os estreptococos do grupo mutans em modelo de células planctônicas e em modelo de biofilme.

2. CAPÍTULOS

Esta tese está de acordo com a Informação CCPG 001/98, UNICAMP, que regulamenta o formato alternativo para dissertação e tese, permitindo a inserção de artigos científicos de autoria ou co-autoria do candidato.

Assim sendo, esta tese é composta de 2 estudos que se encontram em fase de submissão em revistas científicas conforme descrito abaixo:

Estudo 1: **"**Effects of *Mikania* genus plant on growth and cell adherence of mutans streptococci."

Estudo 2: "Antimicrobial activity of compounds isolated from *Mikania sp.* on mutans streptococci biofilms".

O estudo 1 foi submetido a revista Journal of Ethnopharmacology e o estudo 2 será submetido a revista Planta Médica.

O objetivo geral deste trabalho foi analisar *in vitro* o efeito antimicrobiano das plantas *Mikania laevigata* e *Mikania glomerata* sobre os estreptococos do grupo mutans em células planctônicas e em biofilme.

2.1. Estudo 1

Title: Effects of *Mikania* genus plant on growth and cell adherence of mutans streptococci.

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Running title: *Mikania* and mutans streptococci

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SUMMARY

The aim of this study was to evaluate the chemical composition and the antimicrobial activity of the extracts and fractions of *Mikania laevigata* and *Mikania glomerata* on growth and cell adherence of mutans streptococci. Ethanolic extract (EE) and two fractions (hexane-H and ethyl acetate-EA) of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) were chemically identified by chromatographic methods and tested on mutans streptococci from culture collection and clinical isolates. 22 chemical compounds were identified in both *Mikania*, and the main compounds were coumarin, spathulenol, diterpenic, cupressenic and kaurenoic acids. Antimicrobial activity was assessed by determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and inhibition of cell adherence (Adh) to a glass surface. H fraction from both plant extracts was the most effective in inhibiting the growth of all the bacterial strains tested (MIC 12.5 up to 400 µg mL^{-1}, MBC 25 up to 400 µg mL^{-1}). In addition, sub-MIC levels of the crude extracts and their H fractions significantly inhibited the adherence of all microorganisms to a glass surface. Overall, MIC and MBC values for the clinical isolates microorganisms were higher than those obtained from culture collection strains. The data indicate that non polar hexane fraction of both *Mikania* species harbors most of the active compounds, showing remarkable inhibitory activities against mutans streptococci. Mikania genus plant is a promising source for novel antimicrobial agents against oral pathogens.

KEYWORDS: *Mikania*, mutans streptococci, antibacterial, adherence, biofilm.

RUNNING TITLE: Antimicrobial effect of *Mikania* against mutans streptococci.

INTRODUCTION

Natural products have been used for thousand of years in folk medicine, and are promising sources for discovery of novel potentially therapeutic agents (Cragg, 1997; Harvey, 2000). Nowadays they have been used and also investigated more thoroughly as promising agents to prevent oral diseases, especially plaque-related diseases such as dental caries (Wu-Yuan *et al*., 1988; Cai and Wu, 1996; Park *et al*., 1998; Koo *et al*., 1999; Koo *et al*., 2002).

Among various medicinal plants used in folk-medicine in Brazil, *Mikania* genus plant, a sub-scrub creeper of woody branches, known popularly as "guaco" (Celeghini *et al*., 2001), stands out because of its multiple pharmacological properties, especially anti-inflammatory and antimicrobial activities (Ruppelt *et al.*, 1991; Correa, 1942, Pereira *et al.*, 1994; Yatsuda *et al.*, 2001, 2002; de Moura, 2002; Davino, 1989; Paul *et al.*, 2000; Fierro, 1999).

The genus *Mikania* has about 430 species distributed in the tropical areas of Africa, Asia and America (King and Robinson, 1987). In Brazil, the genus is widely distributed, with about 200 described species (Barroso, 1986). The most studied species are *Mikania laevigata* and *Mikania glomerata*. However, only 10% of *Mikania* has been chemically studied (Fabbri *et al*., 1997). Fifteen compounds have been chemically identified and isolated, and the major compounds are coumarin (Vilegas *et al.*, 1997), coumaric acid, sesquiterpenes and diterpenes (Limberger *et al.*, 2001).

Diterpenic compounds and coumaric acid isolated from the *Mikania* fractions exhibited antimicrobial activity against *Micrococcus luteus*, *Staphylococcus aureus* and *Bacillus subitilis* (Beghetti *et al.*, in press). Kaurenoic acid derivatives substituted on carbon -15 showed antimicrobial and antifungal activities against some pathogens as described by Davino *et al.* (1989). Our preliminary studies have shown that crude extracts of *Mikania laevigata* and *M. glomerata* display promising antimicrobial activity against mutans streptococci, showing inhibitory concentrations as low as 25 μg mL^{-1} (Yatsuda *et al*., 2001, 2002).

Mutans streptococci are the main group of microorganism associated with the pathogenesis of dental caries (Loesche, 1986). These bacteria are commonly found in

human dental plaque (Whiley and Beighton, 1998). In addition, mutans streptococci have the ability to adhere and accumulate on the tooth surface by synthesizing extracellular polysaccharides from sucrose (Tanzer *et al*., 1985; Schilling and Bowen, 1992). This sucrose-dependent adherence and the consequent accumulation of cariogenic streptococci are critical for the development of pathogenic dental plaque related to caries (Hamada and Slade, 1980; Loesche, 1986). Therefore, one of the strategies to prevent the formation and development of dental caries is to control the growth and adherence of mutans streptococci.

Considering the antimicrobial potential of *M. laevigata* and *M. glomerata* against oral pathogens, the aim of this study was to evaluate the chemical composition and the antimicrobial activity of extracts and fractions of *M. laevigata* and *M. glomerata* on growth and cell adherence of mutans streptococci.

MATERIALS AND METHODS

Plant material and chemical studies

The aerial parts of *Mikania laevigata* Schultz Bip. ex Baker and *Mikania glomerata* Sprengel were collected from experimental field at the Pluridisciplinar Center of Chemical, Biological and Agricultural Researches - CPQBA/UNICAMP, Campinas, SP, Brazil. Voucher specimen of *M. laevigata* and *M. glomerata* are deposited at Herbarium of Biology Institute of Unicamp at Campinas under number UEC - 102.046 and UEC 102047, respectively. The leaves were allowed to dry under air circulation (40 ^{o}C) for three days and ground for uses.

Extraction and fractionation

The resulting powder (400 g) was submitted to dynamic maceration with 2 L of ethanol: water at (70:30) solution during 3 hours. The extracts were filtered and this procedure was repeated twice. Then, the extracts were concentrated under reduced pressure and liofilizated resulting in 79.1 g of the *M. laevigata* extract (EE-*Ml*) and 58.2 g of the *M. glomerata* extract (EE-*Mg*).

The hexane fractions were prepared using 20 g of the extracts EE-*Ml* and EE-*Mg* using 70 mL hexane for each extract. The extractions were repeated three times in dispersor Ultra-Turrax. The fractions were concentrated using a rotative evaporator obtaining the

hexane fractions: H-*Ml* (2.8 g) and H-*Mg* (2.6 g). The residues of the extractions described above were used to make the ethyl acetate fraction. The fractions of ethyl acetate: EA-*Ml* (2.4 g) and EA-*Mg* (0.32 g).

The hydroalcoholic extracts (EE-*Ml* and EE-*Mg*), hexane fractions (*H-Ml* and H-*Mg*) and the ethyl acetate fractions (EA-*Ml* and EA-*Mg*), were analyzed by Thin Layer Chromatography - TLC using hexane: ethyl acetate 80:20 (detection anisaldehyde solution). The extracts were analyzed by Gas Chromatography coupled with a Mass Detector (GC-MS) (Beghetti *et al.*, in press). Before the GC-MS analysis, the extracts were methylated using diazomethane solution. The analyses for GC-MS were obtained in a Hewlett-Packard 5890 Model II automated gas chromatograph mass spectrometer data system with selective mass detector Hewlett-Packard 5971 equipped with a capillary column J&W Scientific DB-5 (25m x 0.2mmx 0.33 m), with injector 250 ^{o}C and detector 300 ^{o}C. The CG-MS peaks were identified by computer searches in commercial reference libraries. Good spectral matches for some compounds could be found in the Wiley and National Bureau of Standards (NBS) mass spectral library and for comparison with pattern of the isolated compounds previously (kaurenoic acid, cupressenic acid, coumarin, spathulenol).

Antimicrobial assays

Microorganisms[2]

The microorganisms used in this study were *Streptococcus mutans* Ingbritt 1600, *Streptococcus mutans* OMZ 175 and 3 recent clinical isolates (*Strep. mutans* P20, *Strep. mutans* P6 and *Strep. mutans* D1); *Streptococcus sobrinus* 6715 and 3 recent clinical isolates (*Strep. sobrinus* P7, *Strep. sobrinus* S2 and *Strep. sobrinus* S17); and *Streptococcus cricetus* HS-6 and 3 recent clinical isolates (*Strep. cricetus* P12, *Strep. cricetus* S2 and *Strep. cricetus* S38).

[2] The culture collection microorganisms were kindly donated by Center for Oral Biology – University of Rochester, Rochester, NY, USA (Dr. W. H. Bowen). The clinical isolates microorganisms were kindly donated by the Oral Microbiology, FOP/UNICAMP, SP, Brazil (Dr. R. B. Gonçalves).

MIC and MBC Determinations

The antimicrobial activity was determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in accordance with Koo *et al.* (2000) and Duarte *et al.* (2003).

For MIC determination, the starting inoculum was 1-2 x 10^5 cfu mL^{-1}. Two-fold dilution series of extracts or fractions (concentrations ranging from 1.5 to 800 µg mL^{-1} two folds) or negative control (80% ethanol, v/v) were tested. MIC was defined as the lowest concentration of extract or fraction that had restricted growth to a level lower than 0.05 (no visible growth). For the determination of MBC (Minimum Bactericidal Concentration), an aliquot (50 µL) of all incubated test tubes with concentrations higher than the MIC was subcultured on BHI agar supplemented with 5% of desfibrinated sheep blood. The MBC was defined as the lowest concentration that enables no growth on the agar (99.9% kill). Six replicates were made for each concentration of the tested extracts for all assays.

Inhibition of adherence of growing cells to a glass surface

To assess the bacterial adherence of growing cells of mutans streptococci to a glass surface, microorganisms were grown in BHI broth plus 1% sucrose (w/v) as detailed in Koo *et al.* (2000) and Duarte *et al.* (2003). The tubes contained sub-MIC concentrations of crude extracts (EE-*Ml* and EE-*Mg*) and hexane fractions (H-*Ml* and H-*Mg*) of both *Mikania* and negative control (80% ethanol, v/v). After incubation, the adhered cells were washed, resuspended and the adhered cells were spectrophotometrically measured at 550 nm using the procedures outlined by Koo *et al.* (2000). The inhibition of adherence was defined as the lowest concentration that allowed no visible cell adherence on the glass surface. Six replicates were made for each concentration of the tested extracts for all assays.

RESULTS

The chromatograms results of GC-MS of ethanolic extract (EE) of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) are shown in Figure 1 and Table 1. The relative percentages of identified and isolated compounds in EE, hexane fraction (H) and ethyl acetate (EA) of *Ml* and *Mg* are listed in Tables 1, 2 and 3. 22 different chemical compounds in all EE and fractions were found.

It was found that the compounds of the *M. laevigata* were qualitatively similar to *M. glomerata* (Table 1, 2 and 3), except by the coumarin, cupressenic acid, and isobutiloxi-grandifloric that are present only at *M. laevigata* (Table 1, 2 and 3). In the EE-*Ml* (Table 1) the major compounds presents are the octadecen (35.65%), cupressenic acid (27.99%), coumarin (17.81%) and kaurenoic acid (17.64 %) while in EE-*Mg* kaurenoic acid (MM16) (17.94 %) and diterpenic acid (MM 316) (8.08 %) are the main identified compounds (Tables 1). The coumarin in H-*Ml* (40.08%) and the kaurenoic acid (52.47%) in the H-*Mg* are the main identified compounds (Tables 2). In ethyl acetate fraction of *M.* laevigata (EA-*Ml*) (Table 3) the major compound present is cupressenic acid (67.04%) while in *M. glomerata* (EA-*Mg*) the kaurenoic (25.03 %) and diterpenic acid (MM 316) (22.60 %) are the major compounds. Diterpenic ester derived from kaurenoic acid (MM 414) was found only at EE-*Mg* (Table 1) and H-*Mg* (Table 2).

The MIC and MBC values of EE-*Ml*, EE-*Mg*, H-*Ml*, H-*Mg* are shown in Tables 4, and the values of MIC and MBC obtained of EA-*Ml* and EA-*Mg* (200 to 400 μg mL^{-1} MIC and higher than 800 μg mL^{-1} MBC values) were omitted of the Table because not showed biologically effectiveness as hexane fractions. The hexane fraction of both species of *Mikania* (H-*Ml* and H-*Mg*) obtained the lowest MIC and MBC values, showing antimicrobial activities and inhibition of cell adherence against mutans streptococci at concentrations between 12.5 μg mL^{-1} to 100 μg mL^{-1} for MIC (Table 4), 12.5 μg mL^{-1} to 400 μg mL^{-1} for MBC (Table 4), and 3.125 μg mL^{-1} to 12.5 μg mL^{-1} ADH (Table 5).

In the MIC assay (Table 4), all extract and fractions tested showed antimicrobial activity against the tested microorganisms, except against *Strep. mutans* D1 e *Strep. mutans P6* (clinical isolates), which growth was not inhibited only by the crude extract EE-*Ml* and EE-*Mg* at the maximum concentration tested 800 μg mL^{-1}.

In the MBC assay (Table 4), the crude extracts (EE-*Ml* and EE-*Mg*) did not show bactericidal activity against the great majority of clinical isolates microorganisms. Similarly, both EA-*Ml* and EA-*Mg* were not effective against *Strep. sobrinus* and *Strep. cricetus* clinical isolates up to the maximum concentration tested (800 μg mL^{-1}).

The values of the inhibition of mutans streptococci cell adherence to a glass surface obtained by the extracts and the fractions of *M. laevigata* and *M. glomerata* are shown in

Table 5. The values of inhibition were Sub-MIC concentrations; two-three times lower than MIC. The crude extract did not only inhibit the adherence against *Strep. sobrinus* 6715 and *S. mutans* Ingbritt 1600, as well as acted at low concentration (25 µg mL^{-1}). The lowest values of inhibition of cell adherence assays of hexane fraction were between 3.125 and 12.5 µg mL^{-1}. EA-*Ml* and EA-*Mg* did not show any effect at sub-MIC levels.

DISCUSSION

It is well known that mutans streptococci have been considered the major etiological agent in dental caries. Both *Strep. mutans* and *Strep. sobrinus* synthesize extra cellular glucans and fructans from sucrose, which add to the patogenicity of dental plaque (Hamada and Slade, 1980; Whiley and Beighton, 1998).

Therefore, antimicrobial agents against these oral pathogens, as a primary means of controlling bacterial colonization and accumulation of plaque, could play an important part in the prevention of dental caries and periodontal diseases. Thus, it is reasonable to search for natural products that have anti-plaque properties and antimicrobial activity against oral pathogens (Koo *et al.*, 2000).

In this investigation Mikania genus plant showed inhibition of the growth and cell adherence of mutans streptococci. Among all of the crude extracts and fractions tested of both *Mikania*, the hexane fractions were more effective to inhibit mutans streptococci growth, showing MIC values and MBC values at low concentrations. Overall, MIC and MBC values for the clinical isolated microorganisms were higher than those found for culture collection strains, demonstrating that clinical isolated microorganisms are more resistant than laboratory strains, as described by Duarte *et al.* (2003). This data are important because the laboratory strains that are commonly used to determine susceptibility to antimicrobials, may not express the same virulence or resistance level compared with strains recently isolated from the oral cavity (Duarte *et al.*, 2003). On the other hand, hexane fraction was efficient against these bacteria isolated recently.

According to Rios *et al.* (1988), natural crude extracts that have activity at concentrations lower than 100 µg mL^{-1} could have great antimicrobial potential, since the active compounds can be isolated and used at lower concentrations. Our data are relevant

because shows MIC values of hexane fraction of both *Mikania* between 12.5 and 100 $\mu g/mL^{-1}$. Besides, some compounds present in the hexane and ethyl acetate fractions can be responsible for the antimicrobial effect of the EAs fraction, justifying their lower effect than the hexane fraction, where these compounds are in larger quantity.

The compounds cumarina and cupressenic, diterpenic and kaurenoic acids, identified from the hexane fraction (non-polar fraction) of both *Mikania* (Table 2) can be responsible for the greatest antimicrobial activity of this fraction against mutans streptococci in MIC, MBC and adherence cells assays, at lower concentration. It is well established that these compounds isolated are related to antimicrobial activity. Thus, Davino *et al.* (1989) described two kaurenoic acids (kaurenoic acid and cinnamoylgrandifloric acid) have antimicrobial and antifungal activities against some pathogens. Diterpenes from the leaves of *Rabdosia trichocarpa* have been responsible for the antibacterial activity against oral microorganisms (Osawa *et al.*, 1994). Nevertheless, some compounds identified in *Mikania* samples, like cupressenic acid, have not been thoroughly investigated, and little is known about its antimicrobial activity against oral microorganisms or their effects on dental plaque formation *in vitro*.

In addition, the extracts and the fractions of *M. laevigata* and *M. glomerata* also showed *in vitro* inhibition of mutans streptococci cell adherence to a glass surface at lower concentration and sub MIC values. The results expressed in Table 3 showed that the most effective extract against mutans adherence once more was the hexane fraction of both species, confirming the effective of hexane fraction showed by lower MIC and MBC values. The inhibitory effect of these fractions on cell adherence of mutans strains can be related to their action on GTF enzyme, inhibiting glucans production, specially insoluble glucans. GTF enzymes are the main responsible for the pathogenic potential of dental plaque by promoting the adherence and accumulation of mutans streptococci on the teeth (Koo *et al.*, 2000, Duarte *et al.*, 2003). However, further studies need to be conducted to confirm the effect of the isolated compounds of these fractions on activity of individual and purified glucosyltransferase enzymes.

In conclusion, the results of the present study regarding the extracts and the purified fractions (hexane and ethyl acetate) of both species of *Mikania* showed remarkable

inhibitory activities against mutans streptococci growth and cell adherence, specially the hexane fractions that were the most effective extract against all bacterial strains. This finding implies that the pharmacologically active compounds identified from the hexane fractions (Table 2) that have non-polar characteristics, could be involved with the antimicrobial effect against mutans streptococci. Therefore, further studies should be developed in order to test these components alone and in association. In addition, the antimicrobial assay showed that the extracts and the fractions of *M. laevigata* and *M. glomerata* acted at lower concentrations and had remarkable antimicrobial activity against growth and inhibition of cell adherence on mutans streptococci.

This great inhibitory effect of *Mikania*, suggest that this plant can be useful for development of antibacterial agents against oral pathogens, being a promising anti-plaque / anti-caries agent and its identified chemical compounds need to be investigated.

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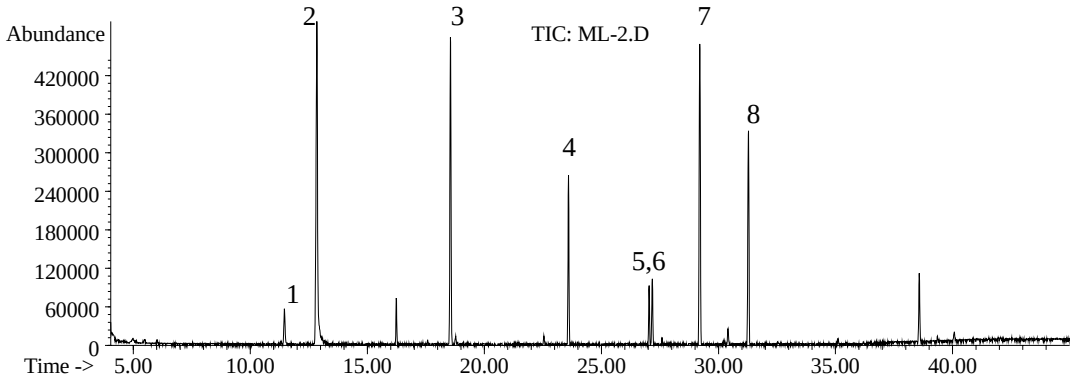
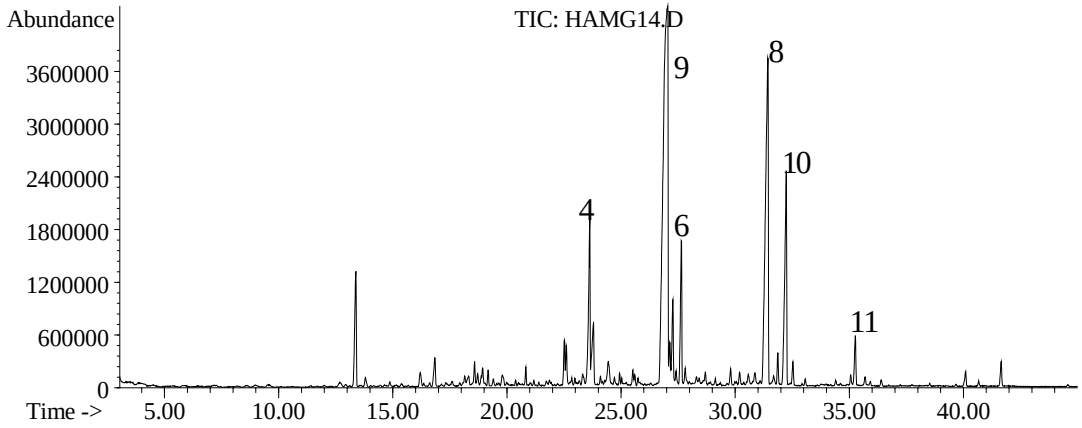
FIGURE

Figure 1– Chromatograms of methylated hydroalcoholic extract of *M. laevigata* and *M. glomerata*.



1= Dihydrocoumarine
2= Coumarine
3= Spathulenol
4= Hexadecanoic acid
5= 9,12-Octadecadienoic acid
6= 9,12,15-Octadecatrineoic acid
7= Cupreseni acid
8= Kaurenoic acid



4= Hexadecanoic acid
6=9,12,15-Octadecatrienoic acid
8= Kaurenoic acid
9= Octadecene
10= Diterpenic acid
11= Grandifloric acid

TABLES

Table 1: Relative percentage of the identified compounds by Gas chromatography (GC-MS) of the ethanolic extract of *M. laevigata* (EE- *Ml*) and *M. glomerata* (EE-*Mg*).

	Isolated and identified compounds	Time. (Min.)	Relative %
Hydroalcoholic extract of *M. laevigata* (EE- *Ml*)	Dihydrocoumarin	11.43	1.93
	Coumarin	12.90	17.81
	Spathulenol	16.19	4.56
	Hexadecanoic acid	23.55	9.23
	Cupressenic acid	29.21	27.99
	Kaurenol	30.32	4.46
	Kaurenoic acid	31.26	17.64
	Isopropiloxi-grandifloric acid	38.51	3.75
	Isobutiloxi-grandiflórico acid	39.99	0.59
	2,5-Ciclohexadiene-1,4-dione,2,6-bis	13.38	3.68
	Hexadecanoic acid	23.62	5.06
	1-Octadecene	27.02	35.65
Hydroalcoholic extract of *M. glomerata* (EE- *Mg*)	9,12,15- Octadecatrienoic acid	27.27	2.37
	Octadecanoic acid	27.64	3.78
	Kaurenoic acid (MM 316)	31.43	17.94
	Diterpenic acid (MM 316)	32.23	8.08
	Grandifloric acid (MM 332)	35.26	1.42
	Isopropiloxi-grandifloric acid	40.10	0.45
	Diterpenic acid (MM 414)	41.65	0.65

Table 2: Relative percentage of the identified compounds by Gas chromatography (GC-MS) of the methylated hexane fraction (H- *Ml*) of *M. laevigata* and *M. glomerata* (H-*Mg*).

	Isolated and identified compounds	Time (Min.)	Relative %
Methylated hexane fraction (H- *Ml*) of *M. laevigata*	Dihydrocoumarin	11.56	1.75
	Coumarin	13.44	40.08
	Spathulenol	16.37	6.17
	Hexadecanoic acid	23.70	9.65
	9,12-Octadecadienoic acid	27.11	3.04
	9,12,15-Octadecatrineoic acid	27.26	3.38
	Cupressenic acid	29.33	7.60
	Kaurenol	30.49	1,83
	Kaurenoic acid	31.39	4.92
	Isopropiloxi-grandifloric acid	38.65	1.40
	Isobutiloxi-grandifloric acid	40.13	0.40
Methylated hexane fraction *(*H- *Mg)* of *M. glomerata*	Spathulenol	12.83	3.03
	Caryophyllene Oxide	12.97	2.84
	Hexadecanoic acid	20.27	12.17
	10,13- Octadecadienoic acid	23.59	6.99
	9,12- Octadecadienoic acid	23.75	10.28
	Kaurenoic acid	27.54	52.47
	Diterpenic acid (MM 316)	28.24	4.13
	Grandifloric acid	31.29	3.42
	Isopropilóxi-grandifloric acid	36.20	1.66
	Diterpenic ester (MM 414)	37.64	2.99

Table 3: Relative percentage of the identified compounds by Gas chromatography (GC-MS) of the methylated ethyl acetate fraction (EA- *Ml*) of *M. laevigata* and *M. glomerata* (EA-*Mg*).

	Isolated and identified compounds	Time (Min.)	Relative %
Methylated ethyl acetate fraction (EA- *Ml*) of *M. laevigata*	Hexadecanoic acid	20.13	10.66
	9,12-Octadecadienoic acid	23.43	2.29
	9,12,15-Octadecatrineoic acid	23.56	3.03
	Cupressenic acid	27.35	67.04
	Kaurenoic acid	28.11	9.62
Methylated ethyl acetate fraction (EA- *Mg*) of *M. glomerata*	Trans-Cariofilene	12.32	2.44
	EI-Bicyclosesquiphellandrene	13.81	4.57
	Spathulenol	16.17	2.50
	Hexadecanoic acid	23.54	7.93
	8,11- Octadecadienoic acid	26.97	4.52
	9,12,15- Octadecatrienoic acid	27.14	11.34
	Kaurenoic acid	31.27	22.60
	Diterpenic acid (MM 316)	32.14	25.03

Table 4: Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of crude extracts (EE-*Ml* and EE-*Mg*), hexane fractions (H-*Ml* and H-*Mg*), and ethyl acetate fractions (EA-*Ml* and EA-*Mg*) of both *Mikania*, against the mutans streptococci strains.

MICROORGANISMS	**Crude extract**				**Hexane**			
	EE-*Ml*		EE-*Mg*		H-*Ml*		H-*Mg*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Strep. mutans Ingbritt 1600	25	50	25	50	12.5	12.5	12.5	25
Strep. mutans OMZ 175	200	400	100	400	100	100	25	400
Strep. mutans D1*	> 800	> 800	> 800	> 800	50	50	12.5	100
Strep. mutans P20*	200	> 800	200	> 800	25	25	25	50
Strep. mutans P6*	> 800	> 800	> 800	> 800	50	50	25	100
Strep. sobrinus 6175	25	50	25	100	12.5	12.5	12.5	25
Strep. sobrinus P7*	200	> 800	200	> 800	12.5	12.5	12.5	50
Strep. sobrinus S2*	100	400	200	400	25	25	12.5	50
Strep. sobrinus S17*	100	> 800	100	> 800	12.5	12.5	12.5	25
Strep. cricetus HS-6	100	100	50	200	12.5	12.5	12.5	200
Strep. cricetus P12*	100	400	200	> 800	25	25	25	50
Strep. cricetus S2*	100	> 800	100	> 800	50	50	25	50
Strep. cricetus S38*	100	> 800	100	> 800	50	50	12.5	50

* Recent clinical isolate microorganism. MIC values are expressed in μg mL^{-1}. The concentrations of extracts and fractions ranged from 1.5- 800 μg mL^{-1}. (n=12)

Table 5: Effect of crude extracts of both *Mikania* (EE-*Ml* and EE-*Mg*) and hexane fractions (H-*Ml* and H-*Mg*) on adherence of growing cells of mutans streptococci strains.

MICROORGANISMS	**Cell Adherence (μg mL^{-1})**			
	EE-*Ml*	**EE-*Mg***	**H-*Ml***	**H-*Mg***
Strep. mutans Ingbritt 1600	25	25	3.125	3.125
Strep. mutans OMZ 175	50	100	6.25	6.25
Strep. mutans P20*	400	400	3.125	3.125
Strep. mutans P6*	400	400	3.125	3.125
Strep. mutans D1*	400	400	3.125	3.125
Strep. sobrinus 6175	25	25	3.125	3.125
Strep. sobrinus P7*	100	100	3.125	3.125
Strep. sobrinus S2*	100	100	3.125	3.125
Strep. sobrinus S17*	100	100	3.125	3.125
Strep. cricetus HS-6	50	50	12.5	12.5
Strep. cricetus P12*	50	100	12.5	12.5
Strep. cricetus S2*	50	100	12.5	12.5
Strep. cricetus S38*	50	100	12.5	12.5

* Recent clinical isolate microorganism.

[a] The Cell adherence values are expressed in μg mL^{-1}. The concentrations of extracts and fractions ranged from 1.5 - 800 μg mL^{-1}. (n=12)

2.2. Estudo 2

Title: "Antimicrobial activity of compounds isolated from *Mikania sp.* on mutans streptococci biofilms".

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ABSTRACT

The aim of this study was to evaluate the influence of the compounds isolated from hexane fractions of *Mikania glomerata* and *Mikania laevigata* on the viability, accumulation and polysaccharide composition of mutans streptococci (*Streptococcus mutans* UA159 and *S. sobrinus* 6715) biofilms. We also explored their influence on the activity of surface-adsorbed glucosyltransferases (GTFs). ent-kaur-16-en-19-oic acid (**1**) and ent-beyer-15-en-19-oic acid (**2**) (from *M. laevigata*), and (**1**) diterpenic acid (**3**) (from *M. glomerata*) were isolated and identified from the hexane fractions by spectroscopic methods. In general, the isolated compounds displayed bactericidal activity against *S. mutans* and *S. sobrinus* biofilms, especially diterpenic acid (at 250 µg/mL) and ent-beyer-15-en-19-oic acid (**2**) (at 500 µg/mL) acids. The biomass (dry-weight) of the biofilms treated with compounds (**3**) (250 µg/mL) and (**2**) (500 µg/mL) acids were significantly less than those treated with vehicle control (10% ethanol, vol./vol.) ($P<0.05$). Furthermore, the insoluble polysaccharide content of the treated-biofilms was significantly affected. Kaurenoic acid exhibited modest inhibitory actions compared to other test agents. Our data showed that compounds from *M. glomerata* and *M. laevigata* were effective against mutans streptococci biofilms; diterpenic acid is a promising novel natural compound for biofilm control.

KEYWORDS: *Mikania sp.*, diterpenic acid, mutans streptococci, biofilm, glucosyltransferases.

INTRODUCTION

The ability of mutans streptococci to produce extracellular polysaccharides, mainly glucans, has been recognized as a critical factor in the pathogenesis of dental caries, and also plaque formation and accumulation (Gibbons and Van Houte, 1975; Hamada e Slade, 1980; Tanzer *et al*., 1985; Loesche, 1986; Yamashita *et al*., 1993). Glucans, synthesized from dietary sucrose by glucosyltransferases (GTFs), are essential for the adherence and accumulation of *Streptococcus mutans* and other oral microorganisms to the tooth surface, leading to the formation of cariogenic biofilm communities (Tanzer *et al.*, 1985; Schilling and Bowen, 1992; Marsh and Bradshaw, 1995). Therefore, mutans streptococci and/or the GTFs should be prime targets for any therapeutic agent aimed at prevention of dental biofilm-related diseases, such as caries and periodontal diseases.

Natural products have been one of the most successful sources for the discovery of novel therapeutic agents (Cragg *et al*., 1997; Harvey 2001). Among various plant extracts used in Brazilian folk-medicine, the ones from *Mikania* genus plant (a sub-scrub creeper of woody branches known popularly as "guaco") are of particular interest due to multiple pharmacological properties, such as anti-inflammatory and antimicrobial activities (Ruppelt *et al.*, 1991; Correa, 1942; Pereira *et al.*, 1994; Celeghini *et al.*, 2001, Yatsuda *et al*., 2001, 2002). Furthermore, our previous studies have shown that hexane fractions of *M. laevigata* and *M. glomerata*, exhibit antimicrobial activity against planktonic cells of mutans streptococci (Yatsuda *et al.*, 2000, 2001). The major components in the hexane fractions were coumarin, and ent-kaur-15-en-19-oic acid (**1**), ent-beyer-15-en-19-oic acid (**2**), diterpenic acid (**3)** and decanoic acid (Yatsuda *et al.*,unpublished).

Recently, we explored the antibacterial activity of several chemical compounds isolated from the hexane fraction of both *Mikania* species. Among them, ent-kaur-16-en-19-oic acid (**1**) and ent-beyer-15-en-19-oic acid (**2**) (from *M. laevigata*), and ent-kaur-16-en-19-oic acid (**1**) and diterpenic acid (**3**) (from *M. glomerata*) inhibited the growth of the planktonic cells of mutans streptococci at low concentrations (MIC 5, 2.5 and 10 µg/mL, respectively (Yatsuda *et al.*, unpublished).

Considering the promising antibacterial effects of the compounds from *M. laevigata* and *M. glomerata*, the purpose of this study was to evaluate the influence of these agents on

the viability, accumulation and polysaccharide composition of mutans streptococci biofilms.

MATERIALS AND METHODS

Plant material and chemical studies

In this study, the compounds from hexane fraction of *M. laevigata (Ml*) and *M. glomerata* (*Mg*) were isolated and identified. The aerial parts of *Mikania laevigata* Schultz Bip. and *Mikania glomerata* Sprengel were collected from Experimental field of the Pluridisciplinar Center of Chemical, Biological and Agricultural Researches - CPQBA/UNICAMP, Campinas, SP. Voucher specimen of *M. laevigata* (*Ml*) and *M. glomerata* (*Mg*) is deposited at Herbarium of Biology Institute of Unicamp at Campinas under number UEC - 102.046 and UEC 102047, respectively.

Extraction and isolation

Dried and pulverized leaves from *M. laevigata* and *M. glomerata (*400 g) were extracted 2 X with ethanol: water (70:30) at room temperature to give 79.1 g and 58.2 g of crude extracts of the *M. laevigata* (EE-*Ml*) and of the *M. glomerata* (EE-*Mg*). 40 g of the extracts were fractionated with hexane for three times in dispersor Ultra-Turrax obtaining the hexane fractions of *M. laevigata* H-*Ml* (5.6 g) and *M. glomerata* H-*Mg* (5.2 g), respectively. The fractions H-*Ml* and H-*Mg* were fractionated separately in dried column chromatography (cellulose 2 X 30 cm) using Si gel 60 with mixtures of chloroform and EtOAc (70:30). The column were cut in four parts and extracted with chloroform supplying 4 fractions: H1-*Ml* (0.45 g), H2-*Ml* (1.80 g), H3-*Ml* (1.86 g), H4-*Ml* (1.50 g); and H1-*Mg* (0.70 g), H2-*Mg* (1.65 g), H3-*Mg* (1.78 g), and H4-*Mg* (1.35 g). The fractions were monitored by TLC using hexane-AcOEt (80:20).

The fraction F2H-*Ml* was purified in column with sílica gel 60 (0.063-0200 mm) impregnated with $AgNO_3$ using hexane:AcOEt in polarity gradient. 72 fractions were collected and monitorated by TLC allowing to isolate 430 mg of the ent-kaur-16-en-19-oic acid (**1**), 195 mg of the ent-beyer-15-en-19-oic acid (**2**) and 68 mg of the mixture of others diterpenic acids.

The fractions H2-*Mg* and H3-*Mg* were grouped and purified on flash column with Si gel 60 (230-400 mesh) by eluting with hexane and gradually increasing the polarity with EtOAc. Sixty fractions were collected and monitorated by TLC. The fraction eluted with hexane-AcOEt (80:20) allowing isolating 350 mg of the ent-kaur-16-en-19-oic acid (**1**) and 64 mg of other diterpenic acid (**3**).

The compounds isolated were crystallized in hexane and those structures were confirmed by comparing physical and spectroscopic properties, including ^{1}H; ^{13}C-NMR and MS spectra, with those reported in the literature; i.e. for (**1)** (Cruz *et al*, 1992., Ohno *et al*, 1979); (**2**) (Takahashi, *et al.*, 2001., Bohlmann *et al.*, 1976); (**3**) (Castro, *et al.*, 1985). Optical rotation was measured with a Perkin-Elmer polarimeter, Melting points (Mp's) were determined on a Koffer boot place and comparison with the data.

Test agents. The concentrations of the isolated compounds used in this study were 1, 10 and 100 times of their MIC values. The biofilms were treated with cupressenic acid (5 µg/mL; 50 µg/mL and 500 µg/mL), diterpenic acid (2.5 µg/mL; 25 µg/mL and 250 µg/mL) and kaurenoic acid (10 µg/mL; 100 µg/mL and 1000 µg/mL). Vehicle control (ethanol 10% v/v) was used as a negative control.

Bacterial strains. The bacterial strains used in this study were *Streptococcus mutans* UA159 and *S. sobrinus* 6715, which are proven virulent cariogenic pathogens (Ajdic *et al.*, 2002). For the production of GTFs, the following constructs were used: *S. milleri* KSB8, which harbors the *gtfB* gene (for GTF B production) and *S. mutans* WHB410, which only expresses *gtfC* gene (for GTF C production).

Preparation of the biofilms. Biofilms were formed on standard glass microscope slides (Micro slides, Glass Técnica Com., São Paulo, SP, Brazil) in batch cultures for 5 days as detailed elsewhere (Ma *et al.*, 1999; Koo *et al.*, 2003). Typically, 5-day-old biofilms yield approximately 10^8 colony forming units (CFU) per mm^2 of slide (Ma *et al.*,

1999).

Time-kill assays. The killing assays were performed according to Koo *et al*. (2003). Briefly, 5 day-old biofilms were exposed to the test compounds (at 1, 10 and 100 times MIC) or vehicle control (10% ethanol, v/v). At specific intervals, the biofilms were removed, suspended and subjected to sonication as detailed in Koo *et al.* (2003). The homogenized suspension was serially diluted (10^{-1} to 10^{-5}) and plated on tryptic soy agar (Oxoid LTD, Basingstoke, UK) by means of a spiral plater (Whitley Automatic Spiral Plater, DW Scientific). The plates were incubated at 10% CO_2 at 37 ^{o}C for 48 h, and then the number of colony forming units per mL (CFU/mL) was determined. Killing curves were constructed by plotting values in the ordinate label, N_0 stands for the original number of CFU/mL and N stands for the number after the indicated times of exposure. All of the assays were carried out in quadruplicate on at least three different times. A bactericidal effect was defined as a > 3 log N/N_0 decreased in the CFU/mL from initial viable counts, at time zero (Koo *et al*., 2002). The potential for drug carryover to produce false low viability counts was minimized by: 1) washing biofilm slides in salt solution (6 times), 2) dilution of inocula, 3) plating small volumes of diluted samples (50 μl).

Inhibition of biofilm formation. The biofilms were grown for 48 h to allow initial bacterial deposition. At this point (48 h old), the biofilms were treated twice daily (one-minute exposure) until the 5th day of the experimental period (120 h-old biofilms) with each of the test compounds (at 1, 10 and 100 times MIC) or vehicle control as described by Koo *et al.* (2003); each biofilm was exposed to the respective treatment six times. Biofilm assays were performed in quadruplicate in at least 3 different experiments. At the end of the experimental period, the biofilms were removed and sonicated; the homogenized suspension was used for dry-weight (biomass), total protein, bacterial viability and polysaccharide analyses as detailed elsewhere (Koo et al., 2003). Total protein of the suspension was determined by the method of Lowry (Lowry *et al*., 1951) using bovine serum albumin to construct the standard curve. For the bacterial viability, an aliquot (0.1 mL) of the homogenized suspension was serially diluted (10^{-1} to 10^{-5}), plated, and the number of CFU determined as described previously. The insoluble polysaccharides were extracted and quantified as detailed elsewhere (Cury *et al.,* 2000; Koo et al., 2003). All of

the assays were carried out in quadruplicate on at least three different times.

Statistical analyses. The data were analyzed using one-way analysis of variance (ANOVA), pairwise comparison was made between all the groups using Tukey-Kramer HSD method to adjust for multiple comparisons, using statistical software JMP version 3.1 (SAS Institute Inc., 1989). When no parametric data was found, Kruskal- Wallis test was used to compare all pairs in the statistical software BioEstat 3.0 (2003). The level of significance was at 5% to both tests.

RESULTS

Figure 1 the chemical structure of the compounds ent-kaur-16-en-19-oic acid (**1**), ent-beyer-15-en-19-oic acid (**2**) isolated from H2-*Ml* while the compounds (**1**) and diterpenic acid (**3**) isolated from H2-*Mg*.

The concentrations used for the test compounds in this study were 1, 10 and 100 times MIC; in general, only the compounds at 100 times MIC displayed significant inhibitory activities compared to control, and their results were shown as follows.

The results of time-kill studies for *S. mutans* UA 159 and *S. sobrinus* 6715 are shown in Figure 2 and 3. Figure 2 shows that diterpenic acid (**3**) and ent-beyer-15-en-19-oic acid (**2**) at 100 times MIC (250 µg/mL and 500 µg/mL respectively) rapidly reduced the viable counts of *S. mutans* within 1 h of treatment (> 1 log N/N_0); after 4 h these compounds showed bactericidal effects (> 3 log N/N_0). The viability of *S. sobrinus* biofilms was also reduced after 4 h (between 1 and 3 log N/N_0) of treatment with tested agents at 100 x MIC. The lower concentration of the agents (1 and 10 x times MIC) showed neglible effects.

For the biofilm accumulation experiment, only the compounds at 100 X MIC showed significant inhibitory effects. The data of the recoverable viable cells from biofilms treated with the test agents (twice daily, one-minute exposure) are shown in Figure 4 and 5. Figure 4 shows that the test agents slightly reduced the number of recoverable viable cells compared to those treated with the vehicle control (0.5 to 0.7 log N/N_0). Similar results were obtained for *S. sobrinus* biofilms as shown in figure 5.

All the agents tested at 100 times MIC significantly reduced the biomass of both *S. mutans* and *S. sobrinus* biofilms compared to control group ($p<0.05$), except to ent-kaur-16-en-19-oic acid (**1**) as shown in Table 1.

Table 1 shows the total amount of insoluble polysaccharide and protein in *S. mutans* biofilms. The diterpenic acid (**3**) (250 µg/mL) was the only agent to significantly reduce the amount of polysaccharide in biofilms compared to control group ($p<0.05$). Ent-beyer-15-en-19-oic acid (**2**) (500 µg/mL) and diterpenic acid (**3**) (250 µg/mL) also reduced the total amount of protein in the biofilms. Regarding *S. sobrinus* biofilms, total amount of polysaccharide was significantly reduced by the ent-beyer-15-en-19-oic acid (**2**) (500 µg/mL) and diterpenic acid (**3**) (250 µg/mL) compared to control group ($p<0.05$); total protein was lower only in biofilms treated with diterpenic acid (**3**) (250 µg/mL). Ent-kaur-16-en-19-oic acid (**1**) was not able to affect significantly the amount of polysaccharide and protein in the biofilms compared to control ($p>0.05$).

DISCUSSION

According to Harvey (2000), the access to biodiversity is fundamental for expanding the range of natural products to be used in the search for new drugs. In this context, *Mikania sp.*, a relatively unexplored natural product, could be a valuable source for exploration of novel bioactive compounds because *Mikania laevigata* and *M. glomerata* extracts showed remarkable inhibitory effects against mutans streptococci (Yatsuda *et al.*, 2001, 2002). In this study, we identified ent-kaur-16-en-19-oic acid (**1**) and ent-beyer-15-en-19-oic acid (**2**) from *M. laevigata* (H2-*Ml*) and diterpenic acid (**3**) and ent-kaur-16-en-19-oic acid (**1**) from *M. glomerata* (H2-*Mg*) as putative novel and active compounds against *S. mutans* and *S. sobrinus* biofilms.

Dental plaque is essentially a biofilm, a population of microorganisms growing on a surface and enclosed in an exopolysaccharide matrix (e.g. glucans) (Lewis, 2001). Furthermore, organisms growing in biofilm mode (sessile bacterial communities) are more resistant than these same organisms in planktonic state (Lewis, 2001).Therefore, it is desirable that potentially novel therapeutic agents aimed at preventing plaque-related oral

diseases present significant influence on the development of the biofilms, and also on glucan-producing GTF enzymes.

The findings of the present study confirm the antimicrobial potential of *Mikania* genus plant; the isolated compounds, cupressenic and diterpenic acids, were effective in reducing the accumulation and insoluble polysaccharide content of mutans streptococci biofilms. Ent-beyer-15-en-19-oic acid (**2**) and diterpenic acid (**3**) are active compounds of *Mikania* extracts.

The diterpenic acid (**3**) was the most effective compound tested reducing both the viability of the biofilms and its insoluble polysaccharide content. This finding is in agreement with in an investigation of diterpenes from the leaves of *Rabdosia trichocarpa* and their antibacterial activity against oral microorganisms, some of these compounds possess potent antibacterial activity, indicating that these diterpenes may be useful natural substances for the maintenance of oral health (Osawa *et al.*, 1994). In addition, Rehder (2001)[1] shows that diterpenic compounds isolated from *Mikania* fractions have antimicrobial activity against some microorganisms, such as *Micrococcus luteus*, *Staphylococcus aureus* and *Bacillus Subitilis.* The ent-beyer-15-en-19-oic acid (**2**) also affected the viability, accumulation and the polysaccharide and protein content of the biofilms. This finding is the first to describe the biological effects of ent-beyer-15-en-19-oic acid (**2**) against microorganisms, and need further investigation. The ent-kaur-16-en-19-oic acid (**1**) exhibited some biological effects against biofilms, although it was not as effect as diterpenic (**3**) and ent-beyer-15-en-19-oic acid (**2**).

Our findings suggest that ent-beyer-15-en-19-oic acid (**2**) and diterpenic (**3**) are promising compounds for development of therapeutic agents for plaque-related diseases. Further studies using *in* vivo models should be conducted in order to evaluate their potential as anti-caries and anti-plaque agents. We are currently pursuing the putative mechanisms of action of these natural compounds.

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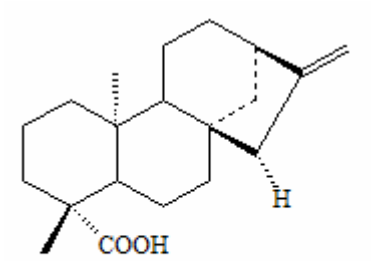
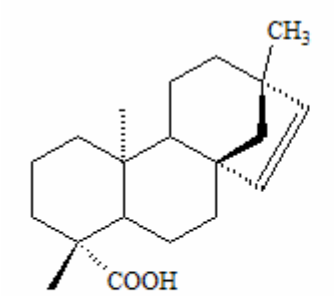
Figure 1: Structures of compounds isolated from *M. laevigata* and *M. glomerata*



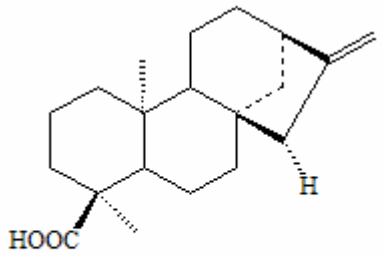
ent-kaur-16-en-19-oic acid

(**1**)



ent-beyer-15-en-19-oic acid

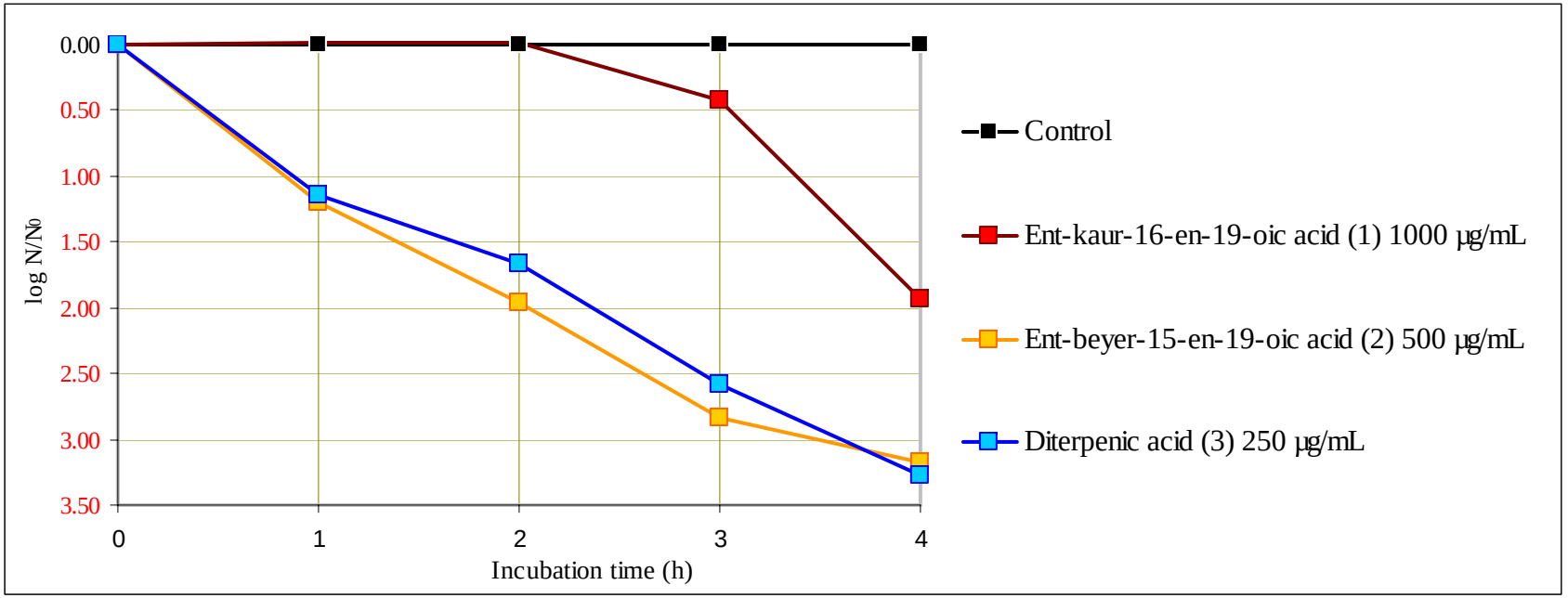
(**2**)



diterpenic acid

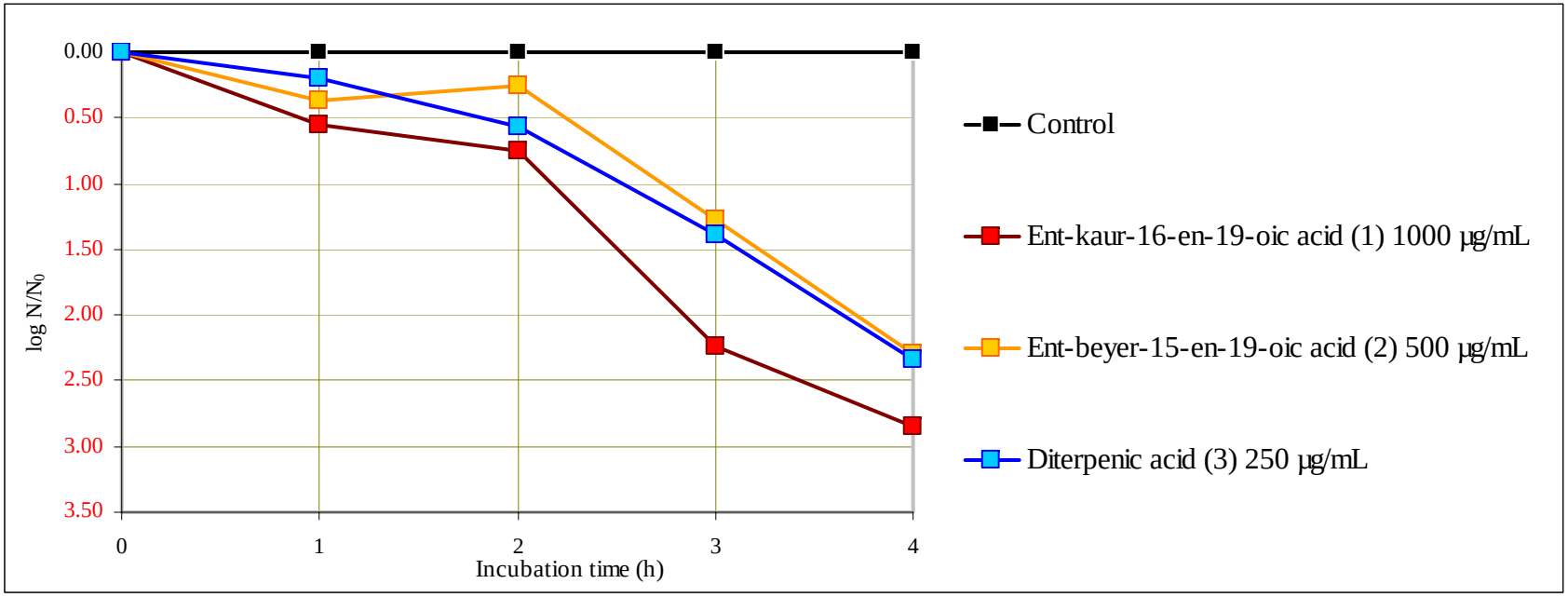
(**3**)

Figure 2. Killing of *S. mutans* UA159 biofilms by the test compounds at 100 times the MIC.



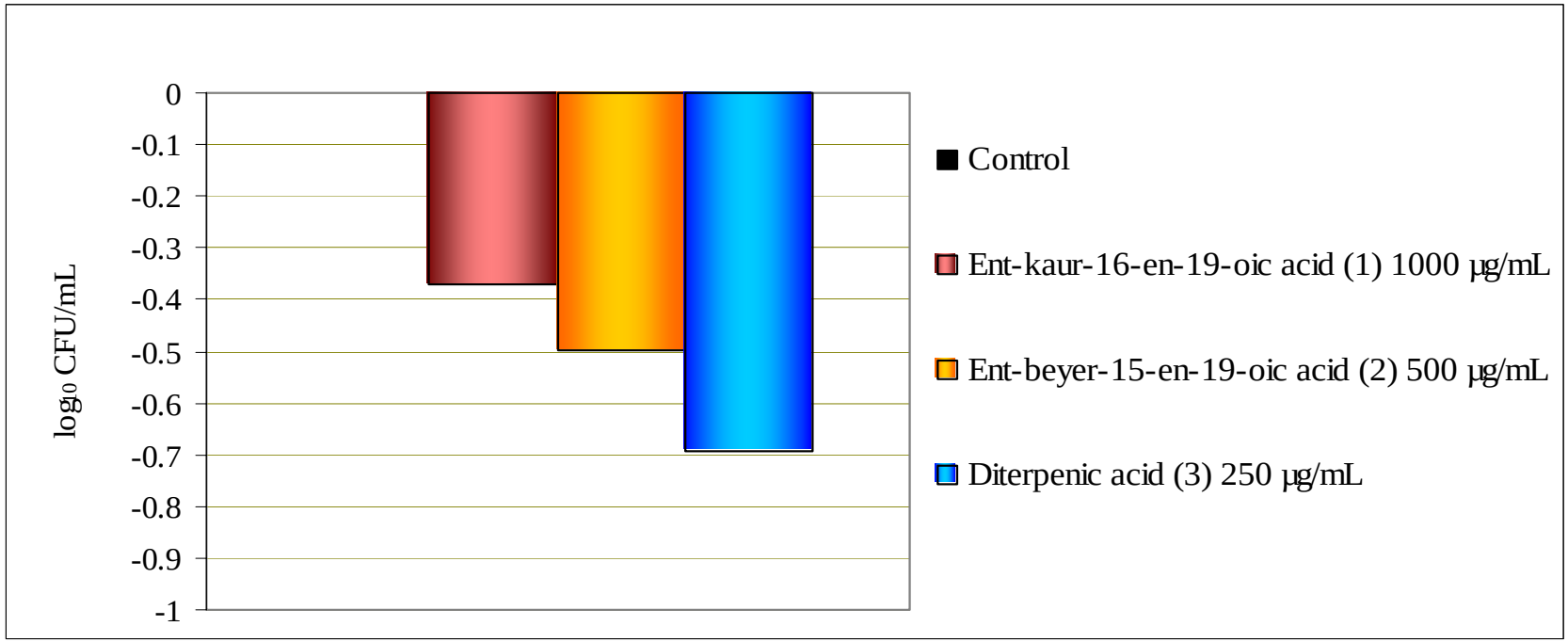
Ent-kaur-16-en-19-oic acid (**1**) 1000 µg/mL, ent-beyer-15-en-19-oic acid (**2**) 500 µg/mL, diterpenic acid (**3**) 250 µg/mL. Vehicle control was used as a negative control. Killing curves were constructed by plotting values in the ordinate label, No stands for the original number of colony – forming units per mL and N stands for the number after the indicated times of exposure. (N=12).

Figure 3. Killing of *S. sobrinus* 6715 biofilms by test compounds at 100 times MIC.



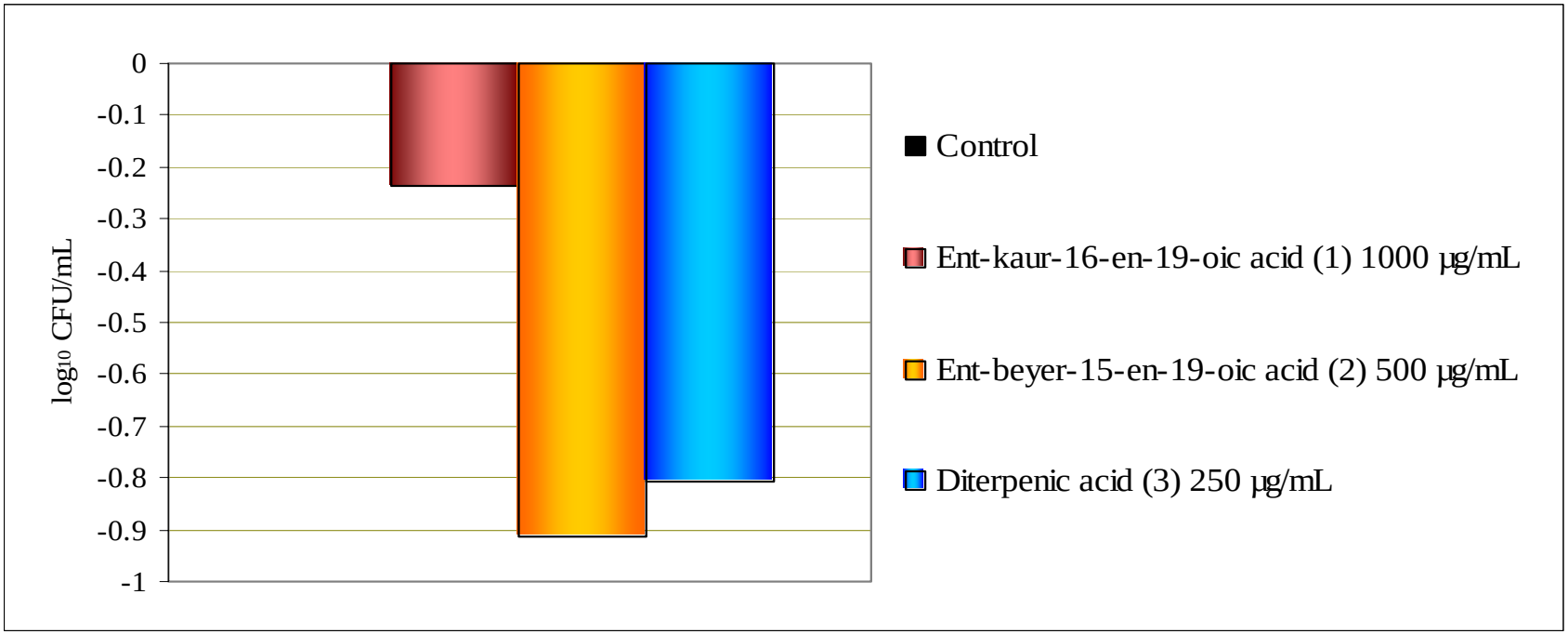
Ent-kaur-16-en-19-oic acid (**1**) 1000 µg/mL, ent-beyer-15-en-19-oic acid (**2**) 500 µg/mL, diterpenic acid (**3**) 250 µg/mL. Vehicle control was used as a negative control. Killing curves were constructed by plotting values in the ordinate label, No stands for the original number of colony – forming units per mL and N stands for the number after the indicated times of exposure. (N=12).

Figure 4. Average number of CFU recovered of the control and after the treatments (120 h old biofilms) per *S. mutans* UA159 biofilms.



Ent-kaur-16-en-19-oic acid (**1**) 1000 µg/mL, ent-beyer-15-en-19-oic acid (**2**) 500 µg/mL, diterpenic acid (**3**) 250 µg/mL. Vehicle control was used as a negative control. Killing curves were constructed by plotting values in the ordinate label, No stands for the original number of colony – forming units per mL and N stands for the number after the indicated times of exposure. (N=12).

Figure 5. Average number of CFU recovered of the control and after the treatments (120 h old biofilms) per *S. sobrinus* 6715 biofilms.



Ent-kaur-16-en-19-oic acid (**1**) 1000 µg/mL, ent-beyer-15-en-19-oic acid (**2**) 500 µg/mL, diterpenic acid (**3**) 250 µg/mL. Vehicle control was used as a negative control. Killing curves were constructed by plotting values in the ordinate label, No stands for the original number of colony – forming units per mL and N stands for the number after the indicated times of exposure. (N=12).

Table 1. Value (means and SD) of dry-weight, total amount of insoluble polysaccharide and protein in the biofilms after treatments.

Treatments	*S. mutans* UA 159			*S. sobrinus* 6715		
	Dry-weight	**Polysaccharide**	**Protein**	**Dry-weight**	**Polysaccharide**	**Protein**
	mg	µg/mg of biofilm	µg/mg of biofilm	mg	µg/mg of biofilm	µg/mg of biofilm
Vehicle control	37.8±3.1[a]	375.7±50.1[a]	274.1±27.4[a]	22.8±2.6[a]	353.1±22.5[a]	325.4±35.5[a]
Ent-kaur-16-en-19-oic acid (**1**) 1000 µg/mL	38.8±1.0[a]	394.2±33.4[a]	275.4±23.3[a]	24.1±2.8[a]	360.4±16.1[a]	373.9±24.3[a]
Ent-beyer-15-en-19-oic acid (**2**) 500 µg/mL	30.1±3.8[b]	325.5±53.8[ab]	211.3±16.7[b]	15.1±3.2[b]	253.9±36.9[b]	369.7±21.6[a]
Diterpenic acid (**3**) 250 µg/mL	31.1±1.3[b]	302.8±38.9[b]	207.5±17.9[b]	14.2±4.1[b]	241.7±14.4[b]	235.3±17.7[b]

Values in the same column followed by the same superscripts are not significantly different from each other (P>0.05, ANOVA, comparison for all pairs using Tukey-Kramer HSD test). (N=12).

3. CONCLUSÕES

Este estudo confirma que a fração hexânica e os compostos isolados da *Mikania laevigata* e *Mikania glomerata,* como os ácidos diterpênico e cupressênico apresentam atividades antimicrobianas contra os estreptococos do grupo mutans em células planctônicas e em modelo de biofilme, demonstrando assim que os compostos isolados da *Mikania* são promissores agentes anti-cárie e anti-placa.

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5. ANEXOS

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